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(54) Title: DETECTION AND TREATMENT OF MUTATIONS IN A CD40 LIGAND GENE (57) Abstract There is disclosed a method of detecting a mutation in a CD40 ligand gene, comprising isolating nucleic acid from an individual, selectively amplifying nucleic acid derived from the CD40 ligand gene, and analyzing the amplified nucleic acid to determine if there is a mutation (or mutations) in the CD40 ligand gene. The ability of a CD40 ligand protein expressed from the gene may be determined. Methods of treating a syndrome that results in elevated levels of serum IgM and diminished levels of all other isotypes of immunoglobulins are also disclosed. Also disclosed are non-human animals that lack functional CD40 ligand as a result of gene targeting technology.		

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TITLE

DETECTION AND TREATMENT OF MUTATIONS IN A CD40 LIGAND GENE

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a ligand for the cell-surface antigen CD40, CD40 ligand (CD40L). More specifically, the present invention relates to methods of detecting mutations in a CD40L gene, and to methods of treating a syndrome that results in elevated levels of serum IgM and diminished levels of all other isotypes of immunoglobulins.

BACKGROUND OF THE INVENTION

Human X-linked hyper-IgM syndrome is characterized by an elevated level of serum IgM and diminished (virtually undetectable) levels of other isotypes of immunoglobulins. Affected males usually experience onset of recurrent infection in the first year of life. The clinical course may include intermittent neutropenia and *Pneumocystis* pneumonia, as well as infections that are more typical of hypogammaglobulinemia, such as bacterial otitis, sinusitis, and pneumonia. This condition is lethal in the absence of medical intervention; however, patients typically respond well to a maintenance therapy consisting of intravenous gamma globulin, especially if therapy is initiated soon after birth.

Affected males have normal numbers of circulating B and T lymphocytes, although lymph node hyperplasia with an absence of germinal centers is common (Notarangelo et al., *Annu. Rev. Immunol.* 10:215, 1992). B-cells from such patients appear to be normal in that they can be induced to undergo isotype switching when cultured in vitro with a T cell line known to induce class switching in normal B-cell cultures (Hendricks et al., *Eur. J. Immunol.* 20:2603, 1990; Mayer et al., *N. Engl. J. Med.* 314:409, 1986).

Elevated levels of serum IgM occur in other syndromes, including combined variable immune deficiency (CVID) and post congenital rubella. Alterations of T-cell activation either as a result of primary genetic immune deficiency or acquired CD4+ T-cell abnormality (e.g. AIDS) may also cause loss of CD40L-induced B-cell activation signals and thus partially explain the secondary abnormalities of B-cell function observed in these conditions.

The CD40 cell surface antigen has been shown to play an important role in B-cell proliferation and differentiation. Human CD40 protein (CD40), a cell-surface antigen present on the surface of B cells, is a peptide of 277 amino acids having a molecular

weight of 30,600, with a 19 amino acid secretory signal peptide comprising predominantly hydrophobic amino acids. A cDNA encoding human CD40 was isolated from a cDNA library prepared from Burkitt lymphoma cell line Raji (Stamenkovic et al., *EMBO J.* 8:1403, 1989).

5 Activated CD4+ T cells express high levels of a ligand for CD40 (CD40L). Human CD40L, a membrane-bound glycoprotein, has recently been cloned from peripheral blood T-cells as described in Spriggs et al., *J.Exp. Med.* 176:1543 (1992), and in United States Patent Application number 07/969,703, filed October 23, 1992, the disclosure of which is incorporated by reference herein. The cloning of murine CD40L is
10 described in Armitage et al., *Nature* 357:80, 1992. CD40L induces B-cell proliferation and secretion of various immunoglobulin isotypes (except IgE) in the absence of any co-stimulus, and can also induce production of IgE in the presence of cytokines.

 CD40L thus appears to play a critical role in the cognate interaction between CD4+ T helper cells and B cells. A more detailed analysis of patients with X-linked hyper IgM
15 syndrome and its related syndromes will provide valuable information on the T cell-B cell interactions involved in the humoral immune response. Early detection of X-linked hyper IgM syndrome and its related syndromes will allow prompt initiation of appropriate therapy. Therefore, there is a need in the art to develop methods of detecting and confirming X-linked hyper IgM syndrome and other abnormalities in B cell-T cell
20 interactions in which CD40 and CD40L play a role. Alternative methods of treatment of such syndromes are also needed.

SUMMARY OF THE INVENTION

 The present invention relates to methods of detecting a mutation or mutations in a
25 CD40L gene, comprising isolating nucleic acid (RNA or DNA) from an individual, selectively amplifying nucleic acid derived from the CD40 ligand gene and analyzing the amplified nucleic acid to determine if there is a mutation (or mutations) in the CD40L gene. Mutations in this gene result in abnormalities in the interaction of CD40L and CD40, which result in alterations in the interactions of T cells and B cells. Such
30 alterations in T cell-B cell interaction play a role in X-linked hyper-IgM syndrome in a human, and may also occur in other syndromes.

 The present invention further provides a method of treating an individual that has a syndrome in which the interaction of T cells and B cells is affected (such as X-linked hyper-IgM syndrome), comprising administering an effective amount of a soluble CD40L.
35 Soluble forms of CD40L comprise the extracellular region of CD40L, and include, for example, fusion proteins comprising the extracellular region of CD40L and an Fc region of a human immunoglobulin, and CD40L multimers formed by adding a multimer-forming peptide to the extracellular region of CD40L.

The present invention also provides a method of utilizing gene therapy to correct X-linked hyper-IgM syndrome and other syndromes in which the CD40L gene does not encode biologically active CD40L. Gene therapy to correct such syndromes comprises isolating CD4+ T cells from an affected individual, transfecting the isolated T cells with a transfection vector that expresses a biologically active CD40L, and administering the transfected T cells expressing biologically active CD40L to the individual.

Also provided are animals that, through gene targeting technology utilizing embryonic stem cells, express non-functional CD40-L in vivo. Such animals, which are referred to as knockout animals, provide a non-human model useful in studying the cognate interaction of T and B cells in vivo.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow chart representing construction of a targeting vector used to generate CD40-L knockout mice. Exons are represented on the map of the CD40-L gene as solid black boxes, and are numbered 1 through 5 from left to right. The solid black arrows represent the PGK-Neo gene; the large open arrows represent the HSV TK gene.

Figure 2 illustrates the gene targeting scheme for pHRV-mCD40L#3.

Figure 3 presents a PCR scheme for genotype determination. PCR primer 3 corresponds to SEQ ID NO:13; PCR primer 4 corresponds to SEQ ID NO:14; PCR primer 1 corresponds to SEQ ID NO:15, and PCR primer 2 corresponds to SEQ ID NO:16.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to syndromes or conditions in which the interaction between T cells and B cells is abnormal due to a defect in a gene encoding a membrane bound ligand for CD40. CD40, a member of the TNF receptor super family, is a membrane-bound receptor protein found to be expressed on B lymphocytes, epithelial cells and some carcinoma cell lines. Monoclonal antibodies directed against CD40 mediate various functional effects of human B cells, including homotypic adhesions, increased cell size, proliferation of B cells activated with anti-IgM, anti-CD20 monoclonal antibody (mAb), phorbol ester alone or phorbol ester combined with interleukin-4 (IL-4), and production of IgE and IgM from IL-4 stimulated, T cell-depleted cultures. These results suggest the importance of CD40 and CD40L in the proliferation and differentiation of B cells.

CD40L is a membrane-bound polypeptide with an extracellular region at its C terminus, a transmembrane region, and an intracellular region at its N-terminus. A soluble version of CD40L can be made from the extracellular region or a fragment thereof. The biological activity of CD40L is mediated by binding to CD40, and comprises proliferation

of B cells and induction of immunoglobulin secretion from activated B cells. CD40L (including soluble oligomeric forms, as well as membrane-bound forms) can effect B cell proliferation and immunoglobulin secretion (except IgE secretion) without the presence of added IL-4, in contrast to anti-CD40 antibodies, which require IL-4 and cross-linking to mediate activity. The cloning of CD40L and certain of its various activities are described

5 in United States Patent Application number 07/969,703, filed October 23, 1992.

The gene for CD40L has been mapped to the proximal region of the murine X chromosome, linked to the *Hprt* locus. In situ hybridization studies of human metaphase chromosomes using a human CD40L cDNA probed confirmed a similar location in

10 humans, with the largest number of grains mapping to band q26. The in vitro biological data on the function of the CD40L together with its chromosomal location suggested that this gene may play a role in an X-linked immunodeficiency. In particular, the phenotype of patients with X-linked hyper-IgM syndrome, and the linkage map position of this syndrome, were most consistent with a defect in the expression of a functional CD40L.

15 X-linked hyper IgM syndrome has been mapped to Xq26, near HPRT (Padayachee et al., *Genomics* 14:551, 1992; Mensink et al., *Hum. Genet.* 76:96, 1987).

Expression of the CD40L gene was examined in patients with primary X-linked hyper-IgM syndrome, using peripheral blood T cells purified by separation over ficoll-hypaque, then activated with immobilized monoclonal antibody to CD3. Soluble CD40

20 protein (a fusion protein containing the extracellular domain of human CD40 fused to the Fc region of human IgG1; referred to herein as CD40.Fc, described in USSN 07/969,703 and in Fanslow et al., *J. Immunol.* 149:655, 1992) was used to analyze the stimulated T cells by fluorescence activated cell sorting (FACS). Control cells (purified T cells from normal adult donors and/or a patient diagnosed with an unrelated X-linked immune

25 deficiency) were analyzed in the same manner. T cells from the X-linked hyper IgM patients failed to express any detectable CD40L upon activation by CD3 antibody (although in some experiments, activated T cells from one patient appeared to weakly bind CD40), but did express the alpha chain of the IL-2 receptor (a T-cell surface activation marker), at levels comparable to that seen on T cells from control donors. T cells from

30 hyper-IgM patients also showed normal proliferative responses to PHA or CD3 mAb plus IL-7.

RNA was extracted from patient and donor cells stimulated as described, and used to generate cDNA which served as a template for PCR reactions. Nucleotide sequence analysis of the resultant cDNAs indicated that single point mutations occurred in the

35 CD40L of three of the four hyper-IgM patients. Each nucleotide change was unique, and all occurred in the extracellular domain of the CD40L. The CD40L cDNA generated from a fourth patient did not appear to contain any nucleotide changes within the coding region,

indicating that another mechanism, possibly involving the 5' or 3' non-coding sequences, must be responsible for the absence of the CD40L on T cells from this patient.

To ensure that these mutations were not merely naturally occurring gene polymorphisms, two of the nucleotide changes found were introduced separately into a mammalian expression vector containing the complete coding region for the human CD40L using site-directed mutagenesis. Cells were transfected with vectors carrying either the wild type or the mutagenized CD40Ls, metabolically radiolabeled, and examined for expression of CD40L protein by precipitation with a polyclonal serum directed against CV1/EBNA cells expressing the human CD40L, or with CD40.Fc. Cells transfected with the wild type CD40L expressed a 33 kD protein that was detected using CD40.Fc. In contrast, cells transfected with either mutant form of CD40L did not express a protein recognized by CD40.Fc. Immunoprecipitation of identical lysates using the polyclonal antiserum resulted in the recognition of a 33 kD protein, from both mutant and wild type transfected cells, which co-migrated with the CD40L protein recognized by CD40.Fc. Northern blot analysis showed similar levels of CD40L-specific RNA in both wild type and mutant transfected cells. Additionally, cells transfected with either mutant form of CD40L were completely negative for CD40.Fc binding, while cells expressing the wild type CD40L showed strong CD40.Fc binding. Cells expressing either form of mutagenized CD40L were also unable to induce B cell proliferation or IgE secretion, confirming the absence of functional CD40L on their cell surfaces.

T cell-depleted peripheral blood mononuclear cells (PBMC; B-cell enriched populations) from X-linked hyper IgM patients showed a proliferative response to CD40L comparable to that seen with similarly purified PBMC from control donors. PBMC from normal donors produced measurable amounts of IgE when cultured with IL-4, whereas no IgE production was detected in cells from any of the hyper-IgM patients cultured under the same conditions. The addition of recombinant CD40L or a CD40 mAb to cultures containing hyper-IgM patients' PBMC restored the the ability of PBMC from three of four patients to secrete IgE.

The results of these studies illustrate the critical role CD40L appears to play in the cognate interaction between CD4+ T helper cells and B cells. This interaction is one of the principal requirements of a successful humoral immune response to most antigens. Further study of hyper-IgM syndrome and related syndromes will provide valuable information on the structural/functional relationship of CD40 and CD40L, and on interactions of the different cells involved in the humoral immune response. Methods of detecting abnormalities in CD40L will provide clarification of the various putative forms (autosomal recessive, autosomal dominant) of hyper-IgM syndrome, as well as other abnormalities in B cell-T cell interactions in which CD40 and CD40L play a role.

Mutations in CD40L can be detected by isolating nucleic acid (DNA or RNA) from an individual, selectively amplifying nucleic acid derived from a CD40L gene, and analyzing the amplified nucleic acid to determine if there is a mutation or mutations in the CD40L gene. Nucleic acids can be isolated from cells such as peripheral blood cells, or fetal cells obtained through amniocentesis or chorionic villus sampling. Other sources of nucleic acids included biopsy tissues, and other tissue samples in which nucleic acids are present.

Nucleic acids may be amplified through polymerase chain reaction (PCR), which utilizes oligonucleotide probes specific for a CD40L gene to selectively hybridize to and amplify those nucleic acids that are derived from the CD40L gene. Oligonucleotide probes that are useful in amplifying RNA derived from the CD40L gene include those probes defined by SEQ ID NOs:1 through 4. Additional oligonucleotide probes derived from the sequence of CD40L gene may be used to analyze the 5' and 3' noncoding regions of the nucleic acid, as well as the sequence of any introns present in the CD40L gene which are not transcribed into mRNA but may affect expression of a functional CD40L gene product.

Nucleic acid may be analyzed to determine if there is a mutation or mutations in a gene by performing nucleotide sequence analysis. Nucleotide sequence analysis may be performed manually, for example by a dideoxy analog chain termination technique. Alternatively, an automated sequencer may be used to perform nucleotide sequence analysis. The nucleotide sequence of the amplified nucleic acid is compared to the published sequence of CD40L (SEQ ID NO:7), which is also disclosed in USSN 07/969,703, the disclosure of which has been incorporated by reference, and Spriggs et al., *J.Exp. Med.* 176:1543 (1992).

Differences in the nucleotide sequence between the amplified nucleic acid and the published sequence of CD40L may result in mutations in a CD40L peptide expressed from the nucleic acid. Such mutations include substitution of a different amino acid (or amino acids) in the CD40L peptide, deletion of one or more amino acids from the CD40L peptide, premature termination of the CD40L peptide, and addition of amino acids to the CD40L peptide. Other types of mutations may cause improper splicing of exons, frame shifts that result in unreadable (nonsense) nucleic acid codons, or affect other elements required for transcription and translation of a biologically active CD40L. Mutations may thus be either in the coding region of a CD40L gene, or in the non-coding regions of the CD40L gene.

The effect of a mutation or mutations on the expression of a biologically active CD40L may be evaluated by introducing the mutation or mutations into an expression vector that encodes biologically active CD40L. A cloning vector containing human CD40L sequence, designated hCD40-L, was deposited with the American Type Culture

Collection, Rockville, MD (ATCC) on December 6, 1991, under accession number 68873. Mutants can be constructed, for example, by using the gene splicing by overlap extension (SOEing) process (Horton et al., *BioTechniques* 8:528, 1990), or by other methods known in the art. The mutant expression vector is then expressed in cells, and the biological activity of the CD40L encoded by the mutant is determined using one or more of the assays for biological activity described herein as well as in USSN 07/969,703, Armitage et al., *Nature* 357:80, 1992, Fanslow et al., *J. Immunol.* 149:655, 1992, and Spriggs et al., *J.Exp. Med.* 176:1543 (1992).

The observation that normal B cell function could be restored by the addition of exogenous, biologically active CD40L supports a therapeutic use of this molecule. Such therapy could comprise administering purified CD40L in a therapeutic composition comprising an effective amount of CD40L in a suitable diluent or carrier. For therapeutic use, purified CD40L or a biologically active analog thereof is administered to a patient, for treatment in a manner appropriate to the indication. CD40L pharmaceutical compositions (for example, in the form of a multimeric soluble extracellular domain, or a fragment thereof) can be given by bolus injection, continuous infusion, sustained release from implants, or other suitable technique.

Typically, a CD40L therapeutic agent will be administered in the form of a pharmaceutical composition comprising purified CD40L polypeptide in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to patients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining a CD40L polypeptide with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents.

A gene encoding biologically active CD40L may also be introduced into T cells (preferably CD4+ T cells) obtained from an individual with abnormal or defective CD40L using gene transfer techniques. The cells are isolated, and transfected with the biologically active CD40L gene; they are subsequently re-administered to the individual, and will then correct the symptoms of the syndrome by producing biologically active CD40L.

Numerous methods have been developed for introducing exogenous genes into mammalian cells, such as by transfection or by infection. These transduction methods may be physical in nature, or they may rely on the use of recombinant retroviral vectors encoding DNA which can be transcribed to RNA, packaged into infectious viral particles and used to infect target cells and thereby deliver the desired genetic material.

Many different types of mammalian gene transfer and expression vectors have been developed (see, Miller and Calos, eds., "Gene Transfer Vectors for Mammalian Cells," *Current Comm. Mol. Biol.*, Cold Spring Harbor Laboratory, New York, 1987). Naked DNA can be physically introduced into mammalian cells by transfection using any
5 one of a number of techniques including, but not limited to, calcium phosphate transfection (Berman et al., *Proc. Natl. Acad. Sci. USA* 84 81: 7176, 1984) DEAE-Dextran transfection, protoplast fusion (Deans et al., *Proc. Natl. Acad. Sci. USA* 84 81: 1292, 1984), electroporation (Potter et al., *Proc. Natl. Acad. Sci. USA* 84 81: 7161, 1984), lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413, 1987), polybrene
10 transfection (Kawai and Nishizawa, *Mol. Cell. Biol* 4:1172, 1984) and direct gene transfer by laser micropuncture of cell membranes (Tao et al., *Proc. Natl. Acad. Sci. USA* 84 84:4180, 1987).

Various infection techniques have been developed which utilize recombinant infectious virus particles for gene delivery. This represents a preferred approach to the
15 present invention. The viral vectors which have been used in this way include virus vectors derived from simian virus 40 (SV40; Karlsson et al., *Proc. Natl. Acad. Sci. USA* 84 82:158, 1985), adenoviruses (Karlsson et al., *EMBO J.* 5:2377, 1986), adeno-associated virus (LaFace et al., *Virology* 162:483, 1988) and retroviruses (Coffin, 1985, p17-71 in Weiss et al (eds.), *RNA Tumor Viruses*, 2nd ed., Vol 2, Cold Spring Harbor
20 Laboratory, New York).

Thus, gene transfer and expression methods are numerous but essentially function to introduce and express genetic material in mammalian cells. Several of the above techniques have been used to transduce hematopoietic or lymphoid cells, including calcium phosphate transfection (Berman et al., *supra*, 1984), protoplast fusion (Deans et al., *supra*, 1984), electroporation (Cann et al., *Oncogene* 3:123, 1988), and infection with
25 recombinant adenovirus (Karlsson et al., *supra*; Ruether et al., *Mol. Cell. Biol.* 6:123, 1986) adeno-associated virus (LaFace et al., *supra*) and retrovirus vectors (Overell et al., *Oncogene* 4:1425, 1989). Primary T lymphocytes have been successfully transduced by electroporation (Cann et al., *supra*, 1988) and by retroviral infection (Nishihara et al.,
30 *Cancer Res.* 48:4730, 1988; Kasid et al., *supra*, 1990).

Gene targeting technology utilizing embryonic stem cells has made it possible to create transgenic mice exhibiting defined changes in a selected gene (Waldman A.S. 1992, *Crit. Rev. Oncol. Hematol.* 12(1):49-64; Huang M. T. 1993, *Lab. Anim. Sci.* 43(2):156-159; Koller and Smithies, 1992, *Annu. Rev. Immunol.* 10:705-730; Joyner A.L. 1991, *Bioessays.* 13(12):649-656; Smithies O., 1993 *Trends Genet.* 9(4):112-116). For
35 example, a probe is prepared from a cDNA of the desired gene, and used to identify the actual gene in a genomic library. The structure of the gene is determined, and the desired change(s) developed. Recombinant DNA technology is used to prepare a vector that will

introduce the desired change(s) in the gene of an embryonic stem cell by homologous recombination.

The altered ES cells are then injected into blastocysts, in which they contribute to the formation of all tissue types, including germ cells. The animals that develop from such blastocysts are referred to as chimeric. When the chimeric animals are bred, germ cells that contain the mutation give rise to animals that lack the functional gene. Such animals are referred to as "knockout" (KO) animals. Many examples of KO mice are known; some of these animals exhibit a phenotype similar to that seen in certain human genetic diseases, and thus serve as useful animal models.

CD40-L KO mice are likely to be of great interest to scientists investigating the cognate interactions between T and B cells in thymus-dependent antibody responses, as well as various aspects of immunoglobulin isotype switching. The role of CD40-L in human X-linked hyper-IgM syndrome indicates that CD40-L KO mice would be a valuable asset for testing possible treatments (i.e. administration of soluble, recombinant ligand) for hyper IgM. Additionally, CD40-L knockout mice are of interest for many different types of investigation, in that these animals have an exquisitely defined genetic defect that is expected to disable one specific cellular interaction necessary for an immune response. Thus, CD40-L KO mice are expected to be useful as models for testing vaccine preparations or immune response modifiers, in defining the role of T cells and B cells in various diseases and syndromes (including infectious disease), and in developing treatments for hyper IgM syndrome and other conditions which result from, or are linked to, abnormality in the interaction of T and B cells.

The following examples are intended to illustrate particular embodiments and not limit the scope of the invention. The pertinent disclosure of all references is incorporated by reference herein.

Example 1: Mapping of the CD40L gene

Using a cDNA corresponding to the coding region of the murine CD40L gene (Armitage et al., *Nature* 357:80, 1992), the chromosomal location of the murine CD40 locus was determined by interspecific backcross analysis using progeny derived from mating of [(C57BL/6J x *Mus spretus*)F1 X C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1100 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, *Trends Genet.* 7:113, 1991). C57BL/6J and *Mus spretus* DNAs were digested with several restriction endonucleases and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse CD40L cDNA probe. The

mapping results indicate that the CD40L gene (*CD40l*) is located in the proximal region of the murine X chromosome, linked to hypoxanthine-guanine phosphoribosyl transferase (*Hprt*), bone morphogenic protein-2b2 (*Bmp-2b2*) and connexin-32 (*Cnx-32*). A description of the probes and RFLPs for the loci linked to *Cd40l* including *Hprt*, *Bmp-2b2*, and *Cnx-32* have been reported previously (M.E. Dickinson et al., *Genomics* 6:505, 1990; J.A. Haefliger et al., *J. Biol. Chem.* 267:2057, 1992). Recombination distances were calculated as described (E.L. Green, in *Genetics and Probability in Animal Breeding Experiments*, Oxford University Press, New York, 1992, pp. 77-113) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns. The determined recombination frequencies place the *Cd40l* gene 1.5 +/- 1.1 centiMorgans distal of the *Hprt* locus. HPRT maps to the q26 region of the human X chromosome, suggesting that the human homologue of the CD40L would also map to this region. This was confirmed by the situ hybridization studies of human metaphase chromosomes using a human CD40L cDNA probe. Human metaphase chromosomes were obtained from lymphocytes of two normal male donors. Hybridization was carried out using a human CD40L probe labeled with ³H to a specific activity of 1 X 10⁶ cpm/μg as described (J.D. Marth et al., *Proc. Natl. Acad. Sci. USA* 83:7400, 1986). The final probe concentration was 0.4 ng/μg of hybridization mixture. The slides were exposed for 5-7 weeks. Chromosomes were identified by Q-banding. Of 115 sites of hybridization scored, 18 (16%) were located on the distal portion of the long arm of the X chromosome. The largest number of grains was at band q26, with no significant hybridization on other human chromosomes.

25 **Example 2: Analysis of CD40L expression on hyper-IgM patient T cells**

Expression of CD40L was examined in four adolescent patients whose clinical and laboratory findings were consistent with primary X-linked hyper-IgM syndrome. Three of these patients represented sporadic cases with no similarly affected male relatives. The fourth patient belonged to a family with a documented three generation pedigree showing a classic X-linked inheritance of hyper IgM syndrome.

The expression of CD40L was examined directly on peripheral blood T cells purified from the four hyper-IgM patients. Resting peripheral blood T cells express undetectable levels of both CD40L mRNA and protein, however, stimulation with antibody specific for CD3 for 16 hours results in a significant increase in the level of mRNA and cell surface protein (Spriggs et al., *J. Exp. Med.* 176:1543, 1992). Therefore, peripheral blood leukocytes were purified from patient or control donor heparinized whole blood by separation over ficoll-hypaque. T cells were activated with

immobilized CD3 mAb and analyzed by flow cytometry using a soluble form of CD40. This soluble CD40 protein (CD40.Fc), which was described in USSN 07/969,703 and in Fanslow et al., *J. Immunol.* 149:655, 1992, is a fusion protein containing the extracellular domain of human CD40 fused to the Fc region of human IgG1. All experiments included control cells from normal adult donors and in the case of Patients 1, 3, and 4, from age-matched unaffected males. Control cells for Patient 2 included an age-matched, race-matched male diagnosed with X-linked agammaglobulinemia (XLA), an unrelated immunodeficiency. In contrast to T cells from control donors, T cells from Patients 2, 3, and 4 failed to express any detectable CD40L upon activation by CD3 antibody. In some experiments, activated T cells from Patient 1 appeared to weakly bind CD40. However, activation of T cells from all 4 patients with immobilized CD3 mAb did result in expression of the alpha chain of the IL-2 receptor (IL-2R α), a common T-cell surface activation marker, at levels comparable to that seen on T cells from control donors. In addition, T cells from hyper-IgM patients showed normal proliferative responses to PHA or CD3 mAb plus IL-7. These data indicate that while hyper-IgM patients' T cells respond normally to mitogens or to activation through their T-cell receptors, they do not express wild type CD40L on their cell surfaces.

Example 3: Nucleotide sequence analysis of CD40L
from hyper-IgM patients

The nucleotide sequence of cDNA from the hyper-IgM patients of Example 2 was analyzed. Peripheral blood leukocytes (PBL) from patients or control donors were purified from heparinized whole blood on ficoll-hypaque. Cells were then stimulated by incubating overnight with immobilized CD3 antibody or PHA. RNA was extracted from the stimulated PBL using RNazol (Biotecx, Houston, TX). Five to ten μ g of total RNA was diluted in 10 μ l water and heated at 68°C for five minutes. To this mixture was added 1 μ l RNasin, 2 μ l 10X Perkin-Elmer PCR buffer, 1 μ l 20 mM dNTPs, 2 μ l random hexamer primer (Pharmacia, Uppsala, Sweden), and 100 U reverse transcriptase. The mixture was incubated at room temperature for 10 minutes, then 37°C for 1 minute, and heat inactivated at 95°C for 5 minutes. cDNA from this reaction was used as a template in PCR using the following reaction conditions: 5 μ l of 10X Perkin-Elmer buffer, 50 μ M dNTPs, 1 μ M primers, 2 μ l of the cDNA reaction, and 1.25 U Taq polymerase (Perkin-Elmer) to a total volume of 50 μ l. The mixture was denatured at 95°C for 5 minutes, and after addition of Taq polymerase, 35 cycles of 55°C for 1 minute, 72°C for 1 minute, and 94°C for 1 minute were performed. Two PCR reactions were performed to cover the entire cDNA. The primers used to amplify the 5' portion of the cDNA were 5'-CCAGAAGATACCATTTTC-3' (SEQ ID NO:1) and 5'-AGCCCACTGTAACACAG-3'

(SEQ ID NO:2). The primers for the 3' portion of the cDNA were 5'-CATGTCATAAGTGAGGC-3' (SEQ ID NO:3) and 5'-CATAAGGAGGATCCTAG-3' (SEQ ID NO:4). PCR fragments were filled in with Klenow (Pharmacia, Uppsala, Sweden), separated on 1.5 % agarose gels, and the purified fragments were ligated into
5 SmaI-cut pTZ19R for sequencing. Double stranded sequencing was performed manually with a Sequenase kit (USB, Cleveland, OH). Automated sequencing was performed on an Applied Biosystems Model 373A sequencer. In these experiments, control cells were provided by either unaffected age-matched controls, or in all cases, at least one normal adult donor. In addition, one adolescent male diagnosed with X-linked
10 lymphoproliferative disorder (XLP), an unrelated immunodeficiency, was included as a control for Patient 1. Nucleotide sequence analysis of the resultant cDNAs indicated that single point mutations occurred in the CD40L of three of the four hyper-IgM patients. To ensure that these changes were not artifacts introduced during PCR amplification, all reactions, including the initial cDNA synthesis reaction, were performed at least in
15 duplicate. Each nucleotide change is unique, and all occur in the extracellular domain of the CD40L. These changes result in the following amino acid changes: a glycine to valine change at position 227 in Patient 1; a leucine to proline change at position 155 in Patient 2; and a threonine to asparagine change at position 211 for Patient 3. The CD40L cDNA generated from Patient 4 did not appear to contain any nucleotide changes within the
20 coding region. Because the biological analysis performed on this patient clearly indicates a lack of functional CD40L, another mechanism, possibly involving the 5' or 3' non-coding sequences, must be responsible for the absence of the CD40L on T cells in Patient 4. No nucleotide changes were found in the CD40L cDNAs from any control samples, which included the XLP patient.

25

Example 4: Site-directed mutagenesis of the CD40L cDNA

To test whether the nucleotide changes detected in Example 3 affected the expression of the CD40L or its ability to bind to CD40, the two nucleotide changes found
30 in Patient 1 and Patient 2 of Example 2 were introduced separately into a mammalian expression vector containing the complete coding region for the human CD40L using site-directed mutagenesis. A cloning vector containing human CD40L sequence, designated hCD40-L, was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on December 6, 1991, under accession number 68873. Mutants were
35 constructed using the gene splicing by overlap extension (SOEing) process (Horton et al., *BioTechniques* 8:528, 1990). The primer used to recreate the mutation found in Patient 1 was 5'-TGCGGGCAACAATCCATTCACCTGGGAGTATTTGAATTTGCAA (SEQ ID NO:5). The primer used to recreate the mutation found in Patient 2 was 5'-

CCATGAGCAACAACCTTGGTAACCCCGGAAAATGGGAAACAGC (SEQ ID NO:6).

The remainder of the necessary primers were generated from the CD40L sequence (described in USSN 07/969,703 and in Armitage et al., *Nature* 357:80, 1992 and Spriggs et al., *J.Exp. Med.* 176:1543, 1992). The resultant vectors were referred to as Mutant 1 and Mutant 2, respectively. The introduction of the appropriate nucleotide change was confirmed in the actual expression vectors by sequence analysis of the entire coding region. The human embryonic kidney cell line, 293, was transfected with vectors carrying either the wild type or the mutagenized CD40Ls, and on day 3 post transfection, cells were metabolically radiolabeled with ³⁵S Trans-label (ICN Radiochemicals, Irvine, CA). Cell lysates were prepared and examined for expression of CD40L protein by precipitation with a polyclonal serum directed against CV1/EBNA cells expressing the human CD40L, or with CD40.Fc. Cells transfected with the wild type CD40L expressed a 33 kD protein that can be readily precipitated using CD40.Fc. In contrast, cells transfected with either mutant form of CD40L did not express a protein recognized by CD40.Fc. Immunoprecipitation of identical lysates using the polyclonal antiserum, however, resulted in the recognition of a 33 kD protein from mutant as well as wild type transfected cells. This protein co-migrated with the CD40L protein recognized by the CD40.Fc and was not present in lysates transfected with vector alone. Consistent with these results, Northern blot analysis showed similar levels of CD40L-specific RNA in both wild type and mutant transfected cells.

Transfected cells were also examined by flow cytometric analysis. Cells transfected with either mutant form of CD40L were completely negative for CD40.Fc binding, while cells expressing the wild type CD40L showed strong CD40.Fc binding. To address the biological activity of the mutant CD40L proteins, cells transfected with wild type or mutagenized CD40Ls were examined for their ability to induce proliferation and IgE secretion from purified tonsil B cells co-cultured with IL-4. In contrast to cells transfected with wild type ligand (Table 1), cells expressing either form of mutagenized CD40L were unable to induce B cell proliferation or IgE secretion, confirming the absence of functional CD40L on their cell surface.

Table 1: Mutagenized CD40 ligands are not biologically active

Number of Transfected Cells		Vector alone	Wild type	Mutant 1	Mutant 2
3 x 10 ⁴	cpm* IgE†	425 ± 93 < 0.3	6463 ± 911 25 ± 2.8	375 ± 122 < 0.3	407 ± 92 < 0.3
1 x 10 ⁴	cpm* IgE†	450 ± 38 < 0.3	4553 ± 405 5.7 ± 2.3	496 ± 170 < 0.3	367 ± 64 < 0.3
3 x 10 ³	cpm* IgE†	414 ± 54 < 0.3	1710 ± 171 1.4 ± 0.4	398 ± 143 < 0.3	501 ± 116 < 0.3

*Tritiated thymidine incorporated by 1 x 10⁵ purified tonsil B cells measured at day 4
†IgE secretion (ng/ml) by 1 x 10⁵ tonsil B cells co-cultured for 10 days with 5 ng/ml IL-4

5

Example 5: Hyper-IgM patient B cells respond normally to wild type CD40L

10 To address the ability of X-linked hyper-IgM B cells to respond to wild type CD40L, proliferation and isotype secretion assays were performed. T-depleted peripheral blood mononuclear cells (PBMC) (B-cell enriched populations) from all four patients from Example 2 showed a proliferative response to CD40L comparable to that seen with similarly purified PBMC from control donors. In contrast, no such proliferative response
15 was seen in the T-cell depleted cultures of PBMC obtained from the XLA patient, consistent with the fact that the XLA disorder is characterized by the virtual absence of circulating, mature B lymphocytes.

Previous work has shown that culture of single donor PBMC in the presence of IL-4 results in the production of IgE (IgE secretion from 1 x 10⁵ unfractionated to T-depleted PBMC was determined following 10 days culture with 5 ng/ml IL-4, together
20 with 200 ng/ml G28-5 antibody (monoclonal antibody to CD40, obtained from Dr. E. A. Clark, University of Washington, Seattle, WA) or 1 x 10⁴ fixed CV1/EBNA cells transfected with vector alone or human CD40L. Preparation of PBMC and determination of secreted IgE concentrations were performed as described in Fanslow et al., *J. Immunol.* 149:655, 1992. Results are expressed as the mean +/- SEM of triplicate
25 cultures. PBMC from normal donors (Controls 1, 3, and 4) produced measurable amounts of IgE when cultured with IL-4 (Table 2). In contrast, no IgE production was detected from any of the four hyper-IgM patients cultured under the same conditions. Significantly, in 3 out of 4 cases (Patients 1, 2, and 4), the addition of recombinant
30 CD40L or the CD40 mAb, G28-5, to cultures containing hyper-IgM patients' PBMC restored their ability to secrete IgE. Similarly, the T-depleted PBMC (B-cell enriched cultures) from these three patients and from all controls examined secreted IgE in the

presence of IL-4 plus either recombinant CD40L or G28-5 antibody (Table 2). In the case of Patient 3, no IgE was detected in PBMC cultured with IL-4 and recombinant CD40L or G28-5 antibody. The reason for these results is unclear. Additional PBMC were not available from this patient; thus, it was not possible to determine whether this lack of response was reproducible or due to experimental variation.

Table 2: PBMC from hyper-IgM patients can secrete IgE

A. Unfractionated PBMC		IgE Secreted*			
		Patient/Control 1	2	3	4
Vector alone	Patient	< 0.3	< 0.3	< 0.3	< 0.3
	Control	8.6 ± 1.2	< 0.3	16.3 ± 2.9	22.9 ± 3.7
CD40 ligand	Patient	43.3 ± 4.8	51.0 ± 12.4	< 0.3	33.5 ± 6.0
	Control	73.4 ± 6.7	< 0.3	48.1 ± 4.4	25.7 ± 4.0
G28-5 mAb	Patient	76.4 ± 6.8	54.7 ± 12.5	< 0.3	55.0 ± 8.6
	Control	87.1 ± 9.8	< 0.3	87.2 ± 5.8	84.4 ± 8.2
B. T-depleted PBMC					
Vector alone	Patient	< 0.3	< 0.3	< 0.3	< 0.3
	Control	< 0.3	< 0.3	< 0.3	< 0.3
CD40 ligand	Patient	39.2 ± 4.4	22.6 ± 3.1	< 0.3	23.1 ± 6.3
	Control	128.4 ± 13.6	< 0.3	55.1 ± 4.6	80.2 ± 8.3
G28-5 mAb	Patient	69.4 ± 6.2	34.4 ± 11.9	< 0.3	45.5 ± 6.0
	Control	146.0 ± 12.2	< 0.3	93.5 ± 6.1	98.0 ± 9.5

*IgE secretion from 1×10^5 purified tonsil B cells was measured after 10 days co-culture with 5 ng/ml IL-4, together with 200 ng/ml G28-5 antibody or with CV-1/EBNA cells transfected with CD40L or vector alone.

Example 6: Nucleotide sequence analysis of the CD40L gene

The nucleotide sequence of the CD40L gene is determined using a genomic library prepared from human cells. Clones from the genomic library are digested with restriction enzymes to form restriction fragments which can be electrophoretically separated. The electrophoretically-separated restriction fragments are analyzed by Southern blot using oligonucleotide probes that are 30 to 40 oligonucleotides in length, prepared from various portions of the coding region of CD40L. Overlapping fragments are determined and sequenced; sequencing is performed on sufficient fragments to encompass the entire coding region of CD40L. PCR probes may be prepared based on the sequences of the regions flanking the intron-exon boundary for all exons. The PCR probes are used in a

similar manner to that described in Example 3, to determine if there are abnormalities in the sequences of any non-coding regions present in CD40L genomic material. Such analysis will be useful, for example, in analyzing genomic material from cells obtained from a fetus through amniocentesis or chorionic villus sampling. The PCR probes
5 derived in this manner will also be useful in analyzing genomic material, as may be necessary when there are no abnormalities detected in the coding region of a CD40L gene.

Example 7: Generation of CD40L knockout mice

10 The type of DNA construct prepared is classified as a "replacement-type" vector in which a positively selectable marker (the neomycin resistance gene driven by the murine PGK-1 promoter) is flanked on both sides by regions homologous to the target gene (CD40-L). Such vectors are designed to replace a wild type allele with the altered version by homologous recombination through a double reciprocal crossover event involving the
15 vector sequences homologous to the target. In contrast, random integration is thought to incorporate the entire molecule. Thus, to boost the efficiency of targeting, a negative selection marker (the HSV thymidine kinase, or TK, gene) was inserted in the vector, at the end one region of homology.

After a targeting vector was introduced into ES cells, a positive-negative selection
20 (PNS) was initiated. The neomycin resistance gene confers resistance to G418, while the product of the HSV TK gene results in the transformation of gancyclovir into a toxic compound. Thus, in theory, only cells undergoing homologous recombination survived both selections. This scheme for vector construction and PNS has been well established (Mansour et. al. 1988, *Nature*. 336: 348-352).

25 To facilitate construction of a gene targeting vector for the CD40L, a murine genomic DNA library constructed from the strain 129SV (Stratagene, Cat#946305) was screened with a radiolabeled murine CD40L cDNA probe containing the complete coding region (Armitage et. al. 1992, *Nature*. 357:80-82, and USSN 07/969,703). Six clones were isolated and two, designated λ_1 and λ_2 , were chosen for further analysis based on
30 evidence that they overlapped and each contained a sizable portion of the gene. A restriction map and exon location and sizes were determined using standard methodology, and are shown in Figure 1.

A 6.8Kb BamH1-Spe1 fragment from λ_2 was subcloned into pGEM11 digested with BamH1+Xba1. The resulting plasmid, p λ_2 -6.8BS, was digested with BamH1 and
35 the ends blunted by treatment with Klenow fragment. A blunted 1.5Kb EcoR1-BamH1 fragment from pPGK/Neo(WT)A- was inserted, creating p6.8Neo. This construct was digested with Xho1, blunted with Klenow fragment, and a 2.0Kb, blunted Hind3-Asp718 fragment from λ_1 was inserted. The resulting plasmid, p6.8NeoHA, was digested with

Xho1 and a 2.0Kb Xho1-Sal1 fragment from pTGX105-9 (containing the HSV TK gene) was inserted to create pHRV-mCD40L#1.

pHRV-mCD40L#1, upon homologous recombination, would result in a CD40L allele lacking only exon 4. Further analysis indicated that this mutation may not have completely abolished function of the CD40-L gene. Therefore, exon 3 was also deleted, in the following manner. pλ2-6.8BS was digested with Spe1 + BamH1 and a resulting 7.7Kb fragment was purified. This was ligated with a 5.5Kb BamH1-Xba1 fragment of pHRV-mCD40L#1 to create pHRV-mCD40L#2. This vector, upon homologous recombination, should result in a mutant CD40L allele lacking both exons 3 and 4.

pHRV-mCD40L#2 was electroporated into embryonic stem cells at 200V and 960μF. Cells were cultured on a feeder layer of gamma-irradiated, neomycin-resistant STO cells expressing Leukemia Inhibitory Factor (LIF). Cells were selected in 175μg/mL G418 + 2mM gancyclovir for approximately 10 days. Clones were then plated singly and analyzed via PCR in pools of 5 clones. PCR was performed with the CD40L specific primer TGX106.19 (5'-GGCAAGGTCAAGCTCATCC-3'; SEQ ID NO:9) in conjunction with the antisense neomycin resistance gene primer, TGX63.20 (5'-GATATTGCTGAAGAGCTTGG-3'; SEQ ID NO:10). A total of 800 doubly resistant clones (680 in the 129SV-derived ES line, AB1, and 120 in the C57BL/6-derived ES line, B22) were screened for homologous recombination events in this manner. No positive clones were identified.

After several unsuccessful attempts with pHRV-mCD40L#2, a third plasmid with a longer region of homology to the 3' end of the CD40-L gene was prepared. pHRV-mCD40L#2 was digested with Xba1 and Xho1, and a 4.7Kb fragment was subcloned into pGEM11 to create pHRV2-4.7XX. A 1.9Kb Hind3-Sal1 fragment was replaced with a 2.7Kb Hind3-Sal1 fragment from λ2. This plasmid was named pHRV2-5.5XS. A 0.7Kb Asp718-Sal1 fragment was replaced with a 10.3Kb Asp718-Sal1 fragment from λ1 to create pHRV2-15XS. This plasmid was subsequently digested with Sal1 and a 2.0Kb Xho1-Sal1 fragment from pTGX105-9 (containing the HSV TK gene) was inserted. This resulted in pHRV-mCD40L#3, which was deposited with the American Type Culture Collection (ATCC) under the conditions of the Budapest Treaty on January 19, 1993, and given ATCC Accession number _____. Homologous recombination with pHRV-mCD40L#3 results in the same mutation as pHRV-mCD40L#2, namely the removal of exons 3 and 4.

pHRV-mCD40L#3 was electroporated and ES cells selected as described previously. PCR screening was performed using the CD40L specific primer TGX124.19 (5'-GTATGTGGCTGAACACCTG-3'; SEQ ID NO:11) and the antisense PGK/Neo primer, TGX53.18 (5'-CTTGTGTAGCGCCAAGTG-3'; SEQ ID NO:12). A total of 1191 doubly resistant clones (760 in AB1, 231 in B22, and 200 in a 129SV-derived ES

line, D3) were obtained. Several positives were identified and subjected to karyotypic analysis, and a clone in the D3 line, D3 CD40L3 #9-72, was found to have a normal karyotype.

5 Targeted mutation of the CD40L gene was verified by genomic Southern blot analysis. Genomic DNAs from D3 CD40L3 #9-72 and wild type D3 were digested with Pst1, electrophoresed in agarose, blotted to nitrocellulose, and probed with a radiolabeled 400bp Xba1-Pst1 fragment which lies just upstream of the 5' terminus of pHRV-mCD40L#3. This probe hybridized with a 9.0Kb Pst1 fragment in the wild type D3 genome. Gene targeting, however, introduced a new Pst1 site (in PGK/Neo) 2.2Kb 3' of
10 the 5' Pst1 site. Thus, only a 2.2Kb Pst1 fragment hybridized with the probe in the D3 CD40L3 #9-72 genome.

D3 CD40L3 #9-72 cells were injected into day 3.5 blastocysts isolated from C57BL/6 mice, which have a black coat color. Injected blastocysts were carried to term in pseudopregnant Swiss Webster female mice. D3 CD40L3 #9-72 was created in ES cells
15 derived from the 129SV strain which has a black agouti (brown) coat color. Chimeric offspring were identified by the presence of a mixed coat color (black and brown). Male chimeras were mated with wild type C57BL/6 females. Germline transmission of the mutant CD40-L allele was identified by the presence of black agouti female offspring. These females are heterozygous at the CD40-L locus.

20 Heterozygous females were mated to wild type male C57BL/6 mice. According to standard Mendelian genetics, 50% of all male offspring are expected to be hemizygous for the CD40-L mutation and thus would be expected to display a hyper-IgM phenotype. Hemizygous males are mated to heterozygous females to perpetuate the strain. Additionally, a congenic 129SV strain exhibiting the mutant CD40-L allele may be
25 developed, for example, by backcrossing heterozygous females to wild type 129SV males. Alternatively, male chimeras that transmit the mutant CD40-L gene efficiently are mated to 129SV females. Germline transmission of the mutant X-chromosome would result in heterozygous 129SV females, which are mated to wild type 129SV male to obtain hemizygous 129SV males exhibiting the mutant CD40-L. The hemizygous male 129SV
30 mice are mated to heterozygous 129SV females to produce homozygous females.

The allelic state (i.e. wild type vs. heterozygous vs. homozygous mutant (hemizygous mutant in males)) is determined with a simple PCR scheme described in Figure 3. DNA from a tissue sample (blood or ear biopsy sample) is subjected to PCR amplification using four primers. TGX157.23 (5'-CCCAAGTGTATGAGCATGTGTGT-
35 3'; SEQ ID NO:13) and TGX156.23 (5'-GTTCTCCACCTAGTCATTCATC-3'; SEQ ID NO:14) are specific to a region on the CD40-L gene just 3' of exon 4, and amplify a 250bp fragment. This region has been deleted in the mutant allele. Neo1 (5'-GCCCTGAATGAACTGCAGGACG-3'; SEQ ID NO:15) and Neo2 (5'-

CACGGGTAGCCAACGCTATGTC-3'; SEQ ID NO:16) are specific for the 3' end of the neomycin resistance gene. These primers amplify a 500bp fragment specific to the mutant allele. The presence of the mutant allele is determined by Southern blot analysis substantially as described above.

- 5 A heterozygous female was obtained, and her allelic state analyzed according to the PCR scheme described above and outlined in Figure 2. She was mated to a wild type C57BL/6 male as previously described. A sample from one potentially hemizygous male offspring thereof was analyzed as described above and outlined in Figure 3; results of the analysis indicated that exons 3 and 4 were absent from the CD40-L gene, indicating that
- 10 the mouse was hemizygous.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: ARMITAGE, RICHARD
DAVISON, BARRY
FANSLow, WILLIAM
10 RENSHAW, BLAIR
SPRIGGS, MELANIE
WIDMER, MICHAEL
- (ii) TITLE OF INVENTION: DETECTION AND TREATMENT OF MUTATIONS
IN A CD40 LIGAND GENE
15
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
20 (A) ADDRESSEE: IMMUNEX CORPORATION
(B) STREET: 51 UNIVERSITY STREET
(C) CITY: SEATTLE
(D) STATE: WASHINGTON
(E) COUNTRY: USA
25 (F) ZIP: 98101
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
30 (C) OPERATING SYSTEM: Apple Operating System 7.1
(D) SOFTWARE: MS Word for Apple 5.1, Version a
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
35 (B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/009,258
40 (B) FILING DATE: 01/22/93
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: PERKINS, PATRICIA ANNE
45 (B) REGISTRATION NUMBER: 34,693
(C) REFERENCE/DOCKET NUMBER: 2810-A
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 2065870430
50 (B) TELEFAX: 2065870606
- (2) INFORMATION FOR SEQ ID NO:1:
- 55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 60 (ii) MOLECULE TYPE: Oligonucleotide
- (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCAGAAGATA CCATTTC

17

10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

15

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

25

AGCCCACTGT AACACAG

17

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

30

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

40

CATGTCATAA GTGAGGC

17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

45

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

55

CATAAGGAGG ATCCTAG

17

(2) INFORMATION FOR SEQ ID NO:5:

60

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: Oligonucleotide

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGCGGGCAAC AATCCATTCA CTTGGGAGTA GTATTTGAAT TGCAA 45

(2) INFORMATION FOR SEQ ID NO:6:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 42 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: Oligonucleotide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCATGAGCAA CAACTTGGTA ACCCCGGAAA ATGGGAAACA GC 42

(2) INFORMATION FOR SEQ ID NO:7:

30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 840 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

45 (A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:

(B) CLONE: CD40-L

(ix) FEATURE:

50 (A) NAME/KEY: CDS
 (B) LOCATION: 46..831

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

55 TGCCACCTTC TCTGCCAGAA GATACCATTT CAACTTTAAC ACAGC ATG ATC GAA 54
 Met Ile Glu
 1

60 ACA TAC AAC CAA ACT TCT CCC CGA TCT GCG GCC ACT GGA CTG CCC ATC 102
 Thr Tyr Asn Gln Thr Ser Pro Arg Ser Ala Ala Thr Gly Leu Pro Ile
 5 10 15

5	AGC	ATG	AAA	ATT	TTT	ATG	TAT	TTA	CTT	ACT	GTT	TTT	CTT	ATC	ACC	CAG	150
	Ser	Met	Lys	Ile	Phe	Met	Tyr	Leu	Leu	Thr	Val	Phe	Leu	Ile	Thr	Gln	35
10	ATG	ATT	GGG	TCA	GCA	CTT	TTT	GCT	GTG	TAT	CTT	CAT	AGA	AGG	TTG	GAC	198
	Met	Ile	Gly	Ser	Ala	Leu	Phe	Ala	Val	Tyr	Leu	His	Arg	Arg	Leu	Asp	50
15	AAG	ATA	GAA	GAT	GAA	AGG	AAT	CTT	CAT	GAA	GAT	TTT	GTA	TTC	ATG	AAA	246
	Lys	Ile	Glu	Asp	Glu	Arg	Asn	Leu	His	Glu	Asp	Phe	Val	Phe	Met	Lys	65
20	ACG	ATA	CAG	AGA	TGC	AAC	ACA	GGA	GAA	AGA	TCC	TTA	TCC	TTA	CTG	AAC	294
	Thr	Ile	Gln	Arg	Cys	Asn	Thr	Gly	Glu	Arg	Ser	Leu	Ser	Leu	Leu	Asn	80
25	TGT	GAG	GAG	ATT	AAA	AGC	CAG	TTT	GAA	GGC	TTT	GTG	AAG	GAT	ATA	ATG	342
	Cys	Glu	Glu	Ile	Lys	Ser	Gln	Phe	Glu	Gly	Phe	Val	Lys	Asp	Ile	Met	95
30	TTA	AAC	AAA	GAG	GAG	ACG	AAG	AAA	GAA	AAC	AGC	TTT	GAA	ATG	CAA	AAA	390
	Leu	Asn	Lys	Glu	Glu	Thr	Lys	Lys	Glu	Asn	Ser	Phe	Glu	Met	Gln	Lys	110
35	GGT	GAT	CAG	AAT	CCT	CAA	ATT	GCG	GCA	CAT	GTC	ATA	AGT	GAG	GCC	AGC	438
	Gly	Asp	Gln	Asn	Pro	Gln	Ile	Ala	Ala	His	Val	Ile	Ser	Glu	Ala	Ser	120
40	AGT	AAA	ACA	ACA	TCT	GTG	TTA	CAG	TGG	GCT	GAA	AAA	GGA	TAC	TAC	ACC	486
	Ser	Lys	Thr	Thr	Ser	Val	Leu	Gln	Trp	Ala	Glu	Lys	Gly	Tyr	Tyr	Thr	135
45	ATG	AGC	AAC	AAC	TTG	GTA	ACC	CTG	GAA	AAT	GGG	AAA	CAG	CTG	ACC	GTT	534
	Met	Ser	Asn	Asn	Leu	Val	Thr	Leu	Glu	Asn	Gly	Lys	Gln	Leu	Thr	Val	150
50	AAA	AGA	CAA	GGA	CTC	TAT	TAT	ATC	TAT	GCC	CAA	GTC	ACC	TTC	TGT	TCC	582
	Lys	Arg	Gln	Gly	Leu	Tyr	Tyr	Ile	Tyr	Ala	Gln	Val	Thr	Phe	Cys	Ser	165
55	AAT	CGG	GAA	GCT	TCG	AGT	CAA	GCT	CCA	TTT	ATA	GCC	AGC	CTC	TGC	CTA	630
	Asn	Arg	Glu	Ala	Ser	Ser	Gln	Ala	Pro	Phe	Ile	Ala	Ser	Leu	Cys	Leu	180
60	AAG	TCC	CCC	GGT	AGA	TTC	GAG	AGA	ATC	TTA	CTC	AGA	GCT	GCA	AAT	ACC	678
	Lys	Ser	Pro	Gly	Arg	Phe	Glu	Arg	Ile	Leu	Leu	Arg	Ala	Ala	Asn	Thr	200
65	CAC	AGT	TCC	GCC	AAA	CCT	TGC	GGG	CAA	CAA	TCC	ATT	CAC	TTG	GGA	GGA	726
	His	Ser	Ser	Ala	Lys	Pro	Cys	Gly	Gln	Gln	Ser	Ile	His	Leu	Gly	Gly	215
70	GTA	TTT	GAA	TTG	CAA	CCA	GGT	GCT	TCG	GTG	TTT	GTC	AAT	GTG	ACT	GAT	774
	Val	Phe	Glu	Leu	Gln	Pro	Gly	Ala	Ser	Val	Phe	Val	Asn	Val	Thr	Asp	230
75	CCA	AGC	CAA	GTG	AGC	CAT	GGC	ACT	GGC	TTC	ACG	TCC	TTT	GGC	TTA	CTC	822
	Pro	Ser	Gln	Val	Ser	His	Gly	Thr	Gly	Phe	Thr	Ser	Phe	Gly	Leu	Leu	245

AAA CTC TGAACAGTGT CA
Lys Leu
260

840

5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 261 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

	Met	Ile	Glu	Thr	Tyr	Asn	Gln	Thr	Ser	Pro	Arg	Ser	Ala	Ala	Thr	Gly	
	1					5				10					15		
20	Leu	Pro	Ile	Ser	Met	Lys	Ile	Phe	Met	Tyr	Leu	Leu	Thr	Val	Phe	Leu	
				20					25					30			
	Ile	Thr	Gln	Met	Ile	Gly	Ser	Ala	Leu	Phe	Ala	Val	Tyr	Leu	His	Arg	
			35					40					45				
25	Arg	Leu	Asp	Lys	Ile	Glu	Asp	Glu	Arg	Asn	Leu	His	Glu	Asp	Phe	Val	
		50					55					60					
	Phe	Met	Lys	Thr	Ile	Gln	Arg	Cys	Asn	Thr	Gly	Glu	Arg	Ser	Leu	Ser	
30		65				70					75					80	
	Leu	Leu	Asn	Cys	Glu	Glu	Ile	Lys	Ser	Gln	Phe	Glu	Gly	Phe	Val	Lys	
				85						90					95		
35	Asp	Ile	Met	Leu	Asn	Lys	Glu	Glu	Thr	Lys	Lys	Glu	Asn	Ser	Phe	Glu	
				100					105					110			
	Met	Gln	Lys	Gly	Asp	Gln	Asn	Pro	Gln	Ile	Ala	Ala	His	Val	Ile	Ser	
40			115					120					125				
	Glu	Ala	Ser	Ser	Lys	Thr	Thr	Ser	Val	Leu	Gln	Trp	Ala	Glu	Lys	Gly	
		130					135					140					
	Tyr	Tyr	Thr	Met	Ser	Asn	Asn	Leu	Val	Thr	Leu	Glu	Asn	Gly	Lys	Gln	
45		145				150					155					160	
	Leu	Thr	Val	Lys	Arg	Gln	Gly	Leu	Tyr	Tyr	Ile	Tyr	Ala	Gln	Val	Thr	
				165					170					175			
50	Phe	Cys	Ser	Asn	Arg	Glu	Ala	Ser	Ser	Gln	Ala	Pro	Phe	Ile	Ala	Ser	
				180					185					190			
	Leu	Cys	Leu	Lys	Ser	Pro	Gly	Arg	Phe	Glu	Arg	Ile	Leu	Leu	Arg	Ala	
			195				200						205				
55	Ala	Asn	Thr	His	Ser	Ser	Ala	Lys	Pro	Cys	Gly	Gln	Gln	Ser	Ile	His	
		210					215					220					
	Leu	Gly	Gly	Val	Phe	Glu	Leu	Gln	Pro	Gly	Ala	Ser	Val	Phe	Val	Asn	
60		225				230					235					240	

Val Thr Asp Pro Ser Gln Val Ser His Gly Thr Gly Phe Thr Ser Phe
245 250 255

5 Gly Leu Leu Lys Leu
260

(2) INFORMATION FOR SEQ ID NO:9:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
15 (ii) MOLECULE TYPE: Oligonucleotide

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGCAAGGTCA AGCTCATCC 19

(2) INFORMATION FOR SEQ ID NO:10:

- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Oligonucleotide

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GATATTGCTG AAGAGCTTGG 20

40 (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
45 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Oligonucleotide

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11

GTATGTGGCT GAACACCTG 19

55 (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
60 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12

CTTGTGTAGC GCCAAGTG

18

10 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13

CCCAAGTGTA TGAGCATGTG TGT

23

25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

30 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14

40 GTTCCTCCAC CTAGTCATTC ATC

23

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

45 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15

55

GCCCTGAATG AACTGCAGGA CG

22

(2) INFORMATION FOR SEQ ID NO:16:

60

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16

10 CACGGGTAGC CAACGCTATG TC

22

CLAIMS

5 What is claimed is:

1. A method of detecting a mutation in a CD40 ligand gene, in an individual at risk for X-linked hyper IgM syndrome, comprising:

- 10 (a) isolating nucleic acid from the individual;
 (b) selectively amplifying nucleic acid derived from the CD40 ligand gene;
 (c) analyzing the amplified nucleic acid to determine if there is a mutation in the CD40 ligand gene; and
 (d) determining if a protein expressed from the CD40 ligand gene binds CD40.

15 2. The method of claim 1, wherein the nucleic acid is isolated from peripheral blood lymphocytes.

 3. The method of claim 1 wherein the nucleic acid is isolated from fetal cells obtained by amniocentesis or chorionic villus sampling.

20

 4. The method of claim 2, wherein the nucleic acid is messenger RNA.

 5. The method of claim 4, wherein the peripheral blood lymphocytes are stimulated with a mitogen prior to analysis of the nucleic acid.

25

 6. The method of claim 1 wherein the nucleic acid is DNA.

7. A method of detecting a mutation in a CD40 ligand gene, in an individual at risk for X-linked hyper IgM syndrome, comprising:

- 30 (a) isolating messenger RNA (mRNA) from the individual;
 (b) generating complementary DNA (cDNA) from the mRNA;
 (c) performing a polymerase chain reaction (PCR) on the cDNA, utilizing oligonucleotide probes derived from the coding region of CD40 ligand, to form amplified DNA that is derived from the CD40 ligand gene;
35 (d) performing nucleotide sequence analysis on the amplified DNA to determine its nucleotide sequence;
 (e) analyzing the nucleotide sequence of the amplified DNA to determine if there is a mutation in the CD40 ligand gene; and
 (f) determining if a protein expressed from the CD40 ligand gene binds CD40.

40

8. The method according to claim 7, wherein the mRNA is isolated from peripheral blood lymphocytes.

5 9. The method according to claim 8, wherein the peripheral blood lymphocytes are stimulated with a mitogen prior to isolating the mRNA.

10. The method according to claim 9, wherein the mitogen comprises an antibody to a cell surface antigen referred to as CD3.

10 11. The method according to claim 10, wherein the oligonucleotide probes are those defined by SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

12. The method according to claim 9, wherein the oligonucleotide probes are those defined by SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

15

13. A method of treating an individual with a mutation in a CD40 ligand gene that prevents expression of biologically active CD40 ligand, comprising:

(a) obtaining CD4+ cells from the individual with a mutation in the CD40 ligand gene;

20 (b) inserting into the CD4+ cells an expression vector encoding a biologically active CD40 ligand; and

(c) infusing the CD4+ cells expressing a biologically active CD40 ligand back into the individual.

25 14. The method of claim 13, wherein the mutation in the CD40 ligand gene has resulted in X-linked hyper-IgM syndrome.

30 15. A method of treating an individual exhibiting an elevated level of serum IgM and diminished levels of other isotypes of immunoglobulins, comprising administering an effective amount of a soluble, multimeric CD40 ligand.

35 16. The method of claim 15, wherein the soluble, multimeric CD40 ligand is selected from the group consisting of a fusion protein comprising the extracellular region of CD40 ligand and an Fc region of a human immunoglobulin, and a CD40 ligand multimer formed by adding a multimer-forming peptide to the extracellular region of CD40 ligand.

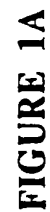
17. The method of claim 16, wherein the elevated level of serum IgM and diminished levels of other isotypes of immunoglobulins are due to hyper-IgM syndrome.

5 18. The method of claim 15, wherein the elevated level of serum IgM and diminished levels of other isotypes of immunoglobulins are due to hyper-IgM syndrome.

19. A non-human animal that, through gene targeting technology utilizing embryonic stem cells, expresses a non-functional CD40 ligand *in vivo*.

10 20. The non-human animal according to claim 19, that lacks exons 3 and 4 of the CD40 ligand gene.

21. The non-human animal according to claim 20, that is obtained by using targeting vector pHRV-mCD40L#3 (ATCC accession number ____).



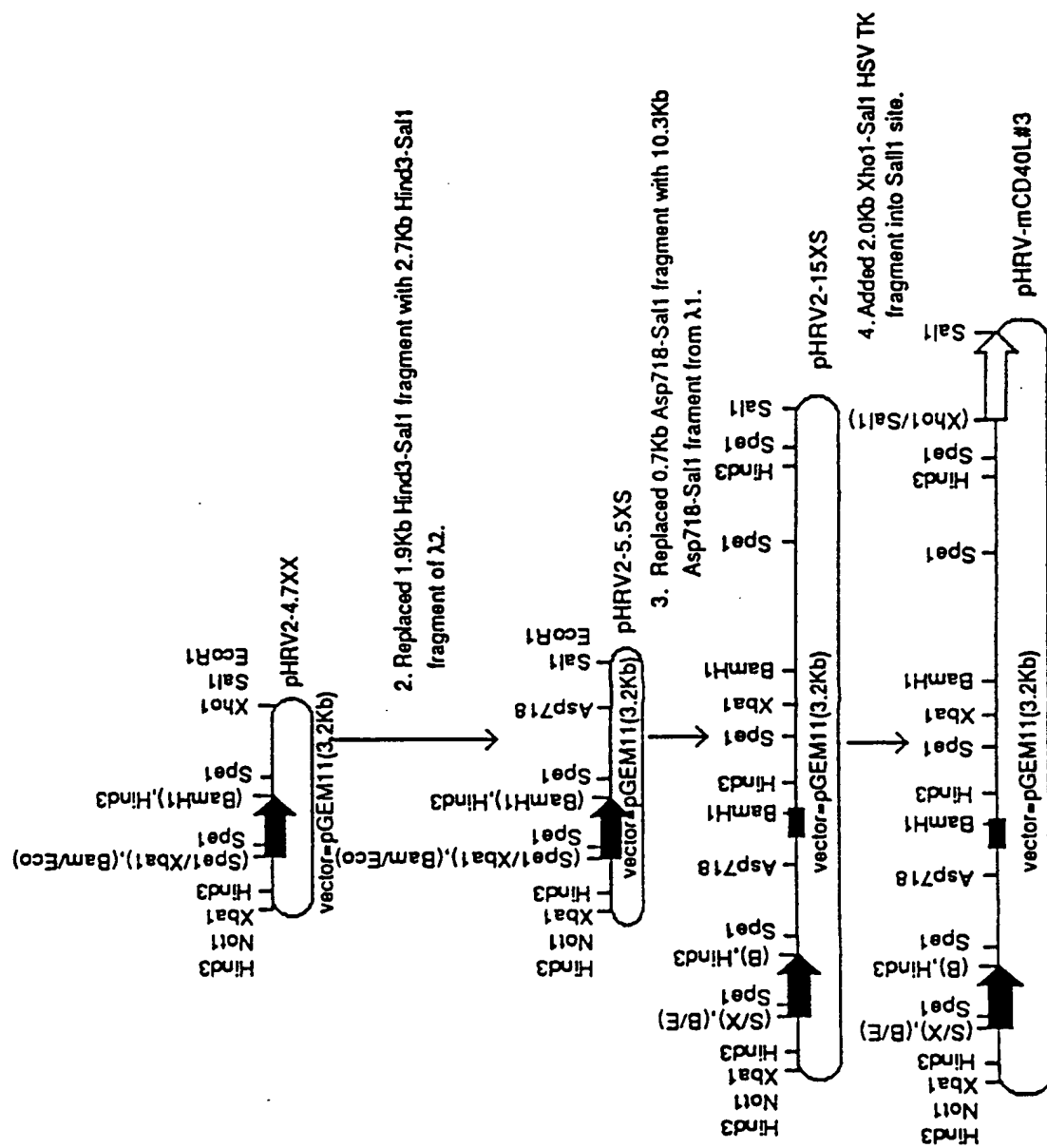


FIGURE 1B

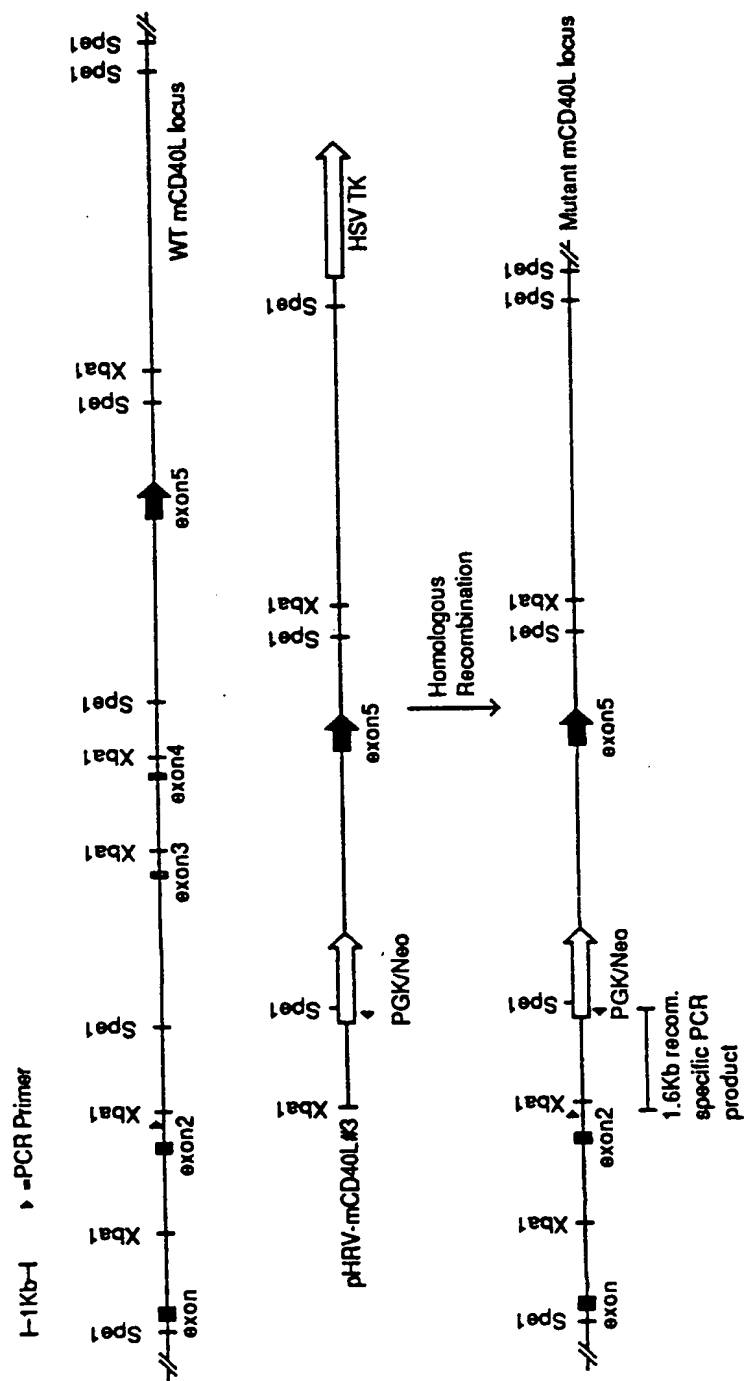


FIGURE 2

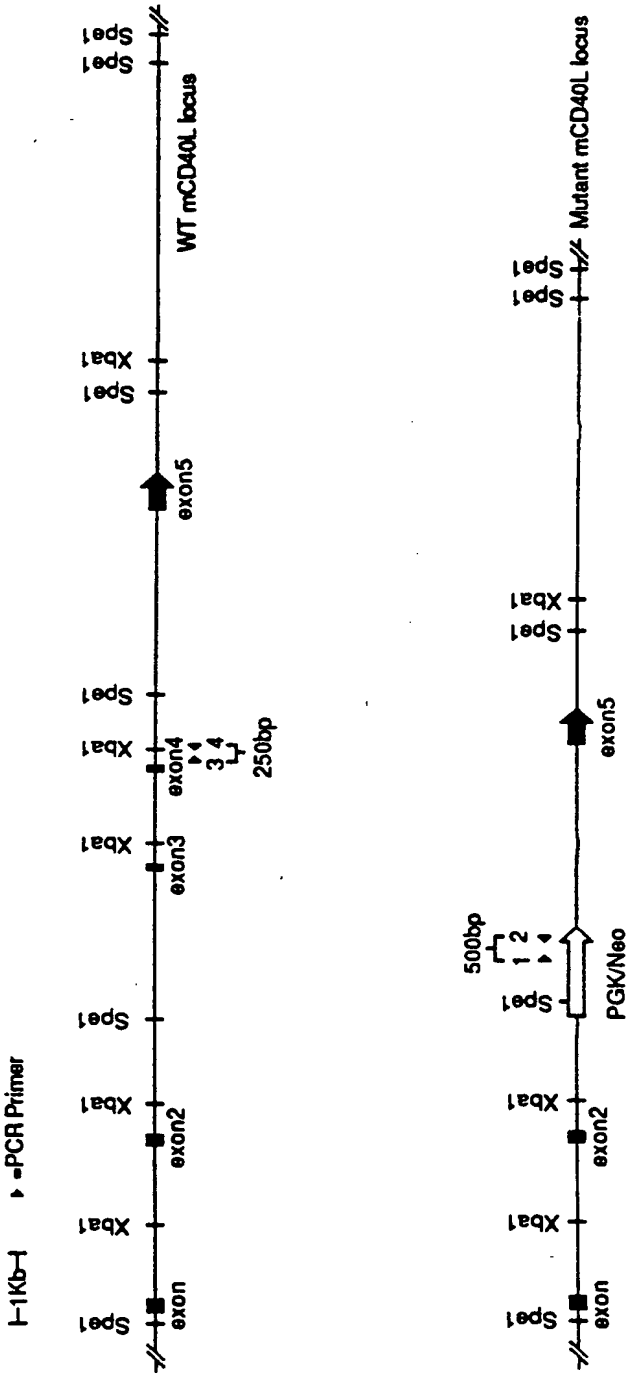


FIGURE 3

INTERNATIONAL SEARCH REPORT

Int. application No.
PCT/US94/00786

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12P 19/34; C12Q 1/70; C07H 21/04; C12N 15/00

US CL : 435/91; 536/23.1, 23.5, 24.3, 24.31; 800/1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91; 536/23.1, 23.5, 24.3, 24.31; 800/1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, EMBASE, MEDLINE, WPI
SEARCH TERMS: CD40 LIGAND, HYPER IGM

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	New Eng. J. Med., Volume 314, No. 7, issued 13 February, 1986, L. Mayer et al., "Evidence for a Defect in Switch T Cells in Patients with Immunodeficiency and Hyperimmunoglobulinemia M", pages 409-413, see entire document.	1-21
Y	Genomics, Volume 14, issued 1992, M. Padayachee et al., "Mapping of the X-Linked Form of Hyper-IgM Syndrome (HIGM1) to Xq26 by Close Linkage to HPRT", pages 551-553, see entire document.	1-21
Y	Eur. J. Immunol., Volume 22, issued 1992, D. Graf et al., "Cloning of TRAP, a Ligand for CD40 on Human T Cells", pages 3191-3194, see entire document.	1-21

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

01 APRIL 1994

Date of mailing of the international search report

14 APR 1994

Name and mailing address of the ISA/US
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Phill Warden for

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00786

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO Journal, Volume 11, No. 12, issued 1992, D. Hollenbaugh et al., "The Human T Cell Antigen gp39, a Member of the TNF Gene Family, Is a Ligand for the CD40 Receptor: Expression of a Soluble Form of gp39 with B Cell Co-Stimulatory Activity", pages 4313-4321, see entire document.	1-21
Y	J. Exp. Med., Volume 176, issued December 1992, M.K. Spriggs et al., "Recombinant Human CD40 Ligand Stimulates B Cell Proliferation and Human Immunoglobulin E Secretion", pages 1543-1550, see entire document.	1-21
Y	J. Immunol., Volume 149, No. 2, issued 15 July 1992, W.C. Fanslow et al., "Soluble Forms of CD40 Inhibit Biologic Responses of Human B Cells", pages 655-660, see entire document.	1-21
Y	US,A 4,873,191 (WAGNER et al.) 10 October 1989, see entire document.	19-21